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Variation in thermally induced melanism in monarch butterflies (Lepidoptera: Nymphalidae) from three North American populations

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Abstract

1. As ectotherms, insects often experience varying temperatures throughout their life cycle, and some respond by becoming more or less melanistic (dark coloring) during development to increase or decrease thermal energy absorption as larvae or adults.
2. Monarch butterflies (*Danaus plexippus*) breed in temperate and tropical environments worldwide and are exposed to different average and extreme temperatures in different parts of their geographic range. In this study, we compared variation in thermally induced melanism among monarch butterflies from eastern and western North America and from South Florida.
3. We raised the progeny of wild-captured adult butterflies from these populations in a common garden experiment, rearing individuals in cold (19 °C), moderate (26 °C), and hot (32 °C) temperatures to examine population variation in larval and adult pigmentation.
4. Across all populations, monarch larvae developed the darkest coloration in the cold treatment and were lightest when reared in hot temperatures. Similar results were observed for measures of adult wing melanism, with the exception of adult females, which developed darker colored wings in warmer temperatures.
5. Significant population-level differences in average measures of melanism among larvae and adult butterflies were observed. Larvae from the eastern population became substantially darker in colder temperatures than S. Florida or western larvae. Western larvae were lightest overall, which might be adaptive to high temperatures experienced throughout portions of their summer breeding range. S. Florida larvae showed a lower response to cold temperatures relative to monarchs from either migratory population.
6. Population level differences were also observed for thermal responses in wing melanism, particularly among adult females. Moreover, we found significant family level effects for each measure of larval and adult melanism, pointing to a genetic basis or strong maternal effects influencing these traits in monarch butterflies.

Keywords: Melanism; *Danaus plexippus*; Population variation; Phenotypic plasticity; Wing coloration

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1. Introduction

Species of insects often occupy multiple climatic regions within their range, and as a result different populations can show local adaptation to their regional environments (Ayres and Scriber, 1994). Local adaptation across a range of temperatures can generate geographical variation in phenotypes within a species, influencing traits such as growth rates and body and wing pigmentation (Ayres and Scriber, 1994; Ellers and Boggs, 2002). For species like butterflies that undergo complete metamorphosis, variation in cuticular melanism of both larval and adult stages might covary with environmental temperatures (e.g. Guppy, 1986). Increased melanization in larvae and adults allows faster absorption of thermal energy, which in turn speeds up larval development (e.g. Goulson, 1994; Hazel, 2002) and facilitates adult flight at lower temperatures (e.g. Pivnick and McNeil, 1986), which allows individuals to occupy a greater range of environments or locations with cooler climates. For example, geographic variation in wing pigmentation as a result of adaptation to local environments has been demonstrated in a number of lepidopteran species (e.g. Berwaerts et al., 1998; Forister and Shapiro, 2003; Ellers and Boggs, 2004), with darker wing coloration generally associated with cooler environments. It important to note that visible changes in response to temperature variation can also result from phenotypic plasticity, or a combination of individual acclimation and genetic change (Kingsolver and Huey, 1998; Hazel, 2002; Solensky and Larkin, 2003; Dombeck and Jaenike, 2004).

Native and introduced monarch butterflies (*Danaus plexippus* L.) populate islands and continents worldwide (Ackery and Vane-Wright, 1984). Despite their wide range, monarch butterflies cannot withstand prolonged freezing temperatures (Calvert et al., 1983), and have exploited temperate resources through the evolution of a spectacular two-way migration in parts of North America and Australia (Urquhart and Urquhart, 1978; James, 1993; Brower, 1995). Each fall in eastern North America monarchs undergo the longest migration, traveling 3500 km or longer from breeding to overwintering sites (Urquhart and Urquhart, 1978; Brower and Malcolm, 1991). In spring, the same butterflies that winter in Mexico mate and fly north to recolonize their breeding range (Van Hook, 1993; Howard and Davis, 2004). A second population in western North America migrates a shorter distance to overwinter along the coast of California (Tuskes and Brower, 1978; Leong, 1990; Frey et al., 1992; Nagano et al., 1993; Brower, 1995). In more tropical areas including S. Florida, Hawaii, Caribbean Islands, Central and South America, monarchs breed year-round and do not migrate (Knight, 1998; Dockx et al., 2004). Aside from different migration strategies, there is little information on evolutionary differences in phenotypic traits among wild monarch populations (but see Altizer, 2001).

While the effects of temperature on monarch butterfly (*D. plexippus*) larval growth, survival and behavior have been well-studied (Zalucki, 1982; James, 1986a; Masters et al., 1988; Masters, 1993; York and Oberhauser, 2002), melanism in monarch butterflies has received less attention. Monarch larvae are characterized by alternating white, yellow and black stripes (Fig. 1A), and the adult butterflies have orange wings bordered with black (Fig. 1B). Black coloring may have thermoregulatory significance in either larvae or adult stages (Masters, 1993). For example, monarch larvae are known to respond to low temperatures by becoming more melanistic (James, 1986b; Solensky and Larkin, 2003; Davis et al., 2004). The larvae accomplish this by

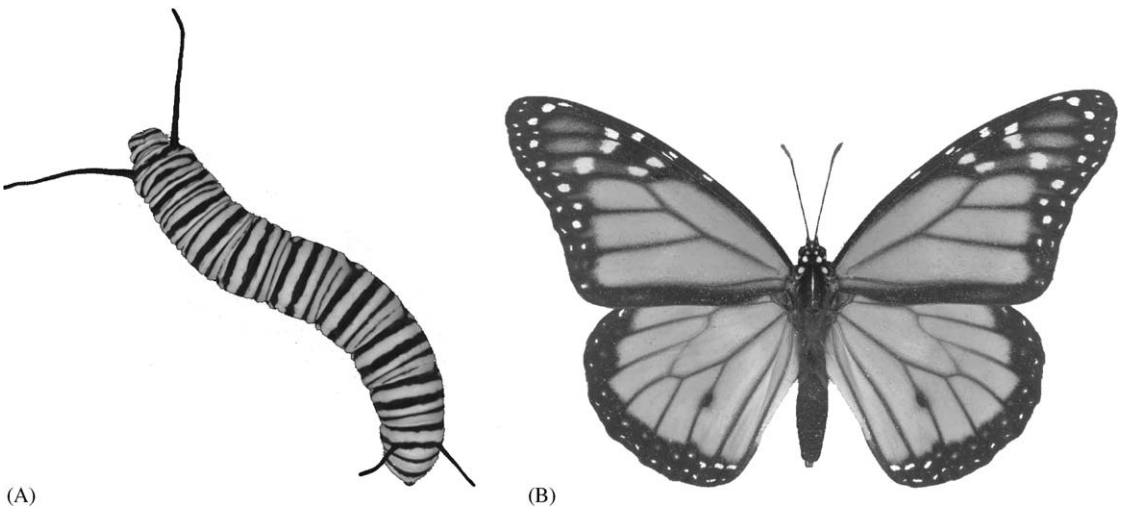

Fig. 1. Typical monarch butterfly larva (fifth instar) and adult male, shown in grayscale.

developing wider black stripes, while the yellow and white stripes shrink (Davis et al., 2004). Because monarch larvae in the wild frequently bask in direct sunlight (Rawlins and Lederhouse, 1981), any changes in the degree of cuticular melanism could directly influence their rates of development by increasing body temperature, which speeds up metabolism (Dixon et al., 1978). Furthermore, early laboratory studies of thermal energy absorption by adult monarchs demonstrated the importance of wings in increasing body temperature (Kammer and Bracchi, 1973). However, overheating may be a problem for larvae and adults in some locations (Masters, 1993), so dark pigmentation in hot climates could be maladaptive.

Monarchs in temperate and tropical populations are likely exposed to different average and extreme temperatures during their larval and adult life stages. In particular, temperatures experienced by larvae and adults from the eastern migratory population are most likely cooler than those experienced by western migratory and Florida resident populations. For example, during their spring northern migration, eastern monarchs oviposit in the gulf coast areas as early as March (Howard and Davis, 2004). Subsequently, their larvae can experience temperatures of 7–20 °C (Climate Diagnostics Center, 2005), corresponding to the lower limit of monarch survival (Masters, 1993). Once in their northern range (upper Midwest, northeastern states and Canada), eastern monarchs undergo several summer generations, where temperatures rarely exceed 27 °C in midsummer (Climate Diagnostics Center, 2005). Moreover, temperatures experienced by eastern adult butterflies at their wintering sites in central Mexico range between 5 and 15 °C (Brower et al., 1977) and can occasionally drop to freezing levels (Brower et al., 2004).

Western monarchs, on the other hand, experience slightly warmer winter temperatures at their overwintering sites, ranging from 5 to 25 °C (Leong et al., 2004). Less is known about the western population's spring movements and summer oviposition habits, making it difficult to predict the range of temperatures these monarchs experience during their breeding season. Wenner and Harris (1993) contended that in the spring, adults oviposit on local milkweed near the coast before spreading north and eastward. Coastal temperatures at this time (March) can range from 8 to 19 °C. Overheating may be more of a problem for western larvae developing later in the summer, particularly in parts of Idaho, Nevada, Utah, California and Arizona (Brower, 1995), where midsummer temperatures frequently reach 32 °C (Climate Diagnostics Center, 2005), close to the upper limit of larval development of 35 °C (Masters, 1993). Finally, larvae in the S. Florida nonmigratory population also must contend with high midsummer temperatures, as maximum temperatures in the Miami area are 32 °C on average (Climate Diagnostics Center, 2005). Winter temperatures in S. Florida are also much milder, with average monthly temperatures from October through February (for Miami) ranging from 19 to 25 °C (Climate Diagnostics Center, 2005).

In light of the range of climates encountered by monarch butterflies in each population, and given the likely relationship between body pigmentation and thermal regulation, it is therefore possible, yet so far untested, that individuals in these three populations would differ in both larval and adult melanism as a result of adaptation to different regional climates. Because the range of temperatures to which monarchs are exposed also differs between populations, each population could also vary in the degree of phenotypically plastic responses to temperature extremes. In this study, we examined thermally induced melanism among monarchs from the three North American populations (eastern, western and S. Florida) in a common garden experiment, rearing individuals from larvae to adulthood in three different temperatures, cold (19 °C), moderate (26 °C), and hot (32 °C). We used two measures of melanism derived using computer-assisted digital image analysis of the monarch subjects—the percentage of area covered by black on the body (larvae) or wing (adults), and the density of the black coloration itself (a measure of the intensity or purity of the black pigmentation). Specifically, we asked the following questions: (1) Do monarchs from each population differ in their overall levels of larval or adult melanism? (2) Do populations differ in their range of plasticity of temperature-induced melanism? (3) Is there a likely genetic basis for variation in melanism levels between and within populations?

2. Methods

Monarchs used in this experiment were the progeny of wild-caught larvae and adults collected from the eastern migratory, western migratory, and S. Florida resident populations during April and May 2003. Monarchs were obtained from two sites in northern Georgia and central Missouri (eastern population; $N = 45$), coastal California (western population; $N = 31$) and Miami and Naples, Florida (S. Florida population; $N = 20$). All wild monarchs were checked for the presence of the protozoan parasite *Ophryocystis elektroscirrha* (Altizer et al., 2000) and infected individuals were removed prior to the experiment. To obtain eggs from these individuals, two 0.6 m^3 mosquito net cages were set up for each population (6 cages in total), and males and females were added so that potential full-sib males and females (based on the site and timing of collection) were assigned to different cages. Cages were monitored each day and the identities of monarchs in all mating pairs were recorded. Following successful mating, females

were held for 24–72 h prior to release into an individual 0.6 m^3 cage with a single potted milkweed plant (*Asclepias incarnata*). After 50 or more eggs were laid on a plant, the plant was removed, labeled with the female's identity and population, and placed near a window at room temperature. We continued to collect eggs until all mated females had oviposited, including a total of 15 females from the eastern migratory population, 10 females from the western migratory population, and 6 females from the S. Florida population.

Plants and eggs were monitored daily for hatching. On the day that eggs began hatching, 36 first instar larvae from each female (or ready-to-hatch eggs as determined by head capsules visible through the egg cuticle) were divided evenly into three treatment groups. These progeny from each female were transferred into plastic containers (0.74 L with small holes punched in the lids) with 3–4 *A. incarnata* leaves and labeled with the female number, temperature treatment, and hatch date. Containers were placed into one of three controlled environment chambers (Conviron Model E15) set to constant temperatures of 19 °C (cold treatment), 26 °C (moderate treatment), and 32 °C (hot treatment). Because previous studies indicated that 26 °C is an optimal temperature for monarch development and survival (Zalucki, 1982), we selected this as our moderate temperature treatment. Past work has shown that temperatures above 33 °C and below 19 °C can result in high pre-adult mortality (Zalucki, 1982; Solensky and Larkin, 2003). Each chamber was kept at a constant light cycle of 12 h light:12 h dark.

A total of 1065 larvae were used to initiate the experiment. Throughout the experiment all containers were checked twice daily and fresh milkweed cuttings (greenhouse reared *A. incarnata*) and paper towel linings were added. Once larvae reached late second instar, they were transferred to larger plastic containers (3.8 L) with wire mesh tops to allow greater air circulation. Larvae in containers with more than 10 individuals were separated into two replicate containers prior to entering fifth instar. To measure larval melanism, all larvae were photographed at fifth instar and dark pigmentation was assessed as described below. For each emerging adult butterfly, we recorded the eclosion date and presence of wing deformities. Adults were placed immediately into glassine envelopes 4–8 h after eclosion and held at room temperature for 24 h prior to being placed in an incubator set to 12 °C. Live adult butterflies were individually scanned to quantify wing pigmentation as described below.

2.1. Acquiring images

To measure melanism levels in monarch larvae and adults, we used a computer-assisted digital image analysis protocol, whereby digital images of the subjects were obtained and computer software was used to generate data. To acquire the images of larvae, we placed fifth instar larvae singly on a light blue cardboard platform and photographed them using an Olympus C-3000 Zoom digital camera that was mounted 25 cm above the platform (following Davis et al., 2004). For adults, we individually scanned live butterflies (after chilling them on ice for 10 min) using an HP flatbed scanner set to 300 dpi. Wings of live butterflies were configured in a standard pinning position using weights to immobilize each monarch. Once all images were obtained, we imported them into Adobe Photoshop with the Image Processing Tool Kit (IPTK) plugin installed (Reindeer Graphics, Inc.). For larvae images, we digitally cropped the tentacles from the body and removed the background prior to image analysis. From the scanned image of the entire adult butterfly, we digitally cropped the right forewing from the body and saved it separately prior to analysis.

2.2. Melanism measurements

We obtained two measures of melanism for each larval and adult image: (1) percentage area of black pigmentation, and (2) density of black pigmentation. Before measurements were initiated, the image analysis program was first calibrated with the image of a standard ruler obtained from the same camera and scanner setup for larvae and adults. Measuring the black pigmentation of each image first required a thresholding step to produce a pure black and white image (so that all non-black colors became white). We then ran the IPTK measurement function to quantify the area (in mm^2) of all black versus white polygons on the resulting image. The percentage of the entire body (larvae) or forewing (adults) encompassed by black was then calculated based on the area of black versus white polygons obtained by the program. To measure the black density (i.e., level of opacity or intensity of the black pigmentation), we extracted only the solid black areas as determined by the thresholding step. The density of black was calculated by overlaying the original color of these black areas (from the original images) onto the solid black areas we defined in the last step and calculating the average pixel density of this black area. Because the computer measures pixel density on a 0–255 scale, with 0 being completely black and 255 being completely white, low density scores actually correspond to darker areas in this scoring system.

2.3. Data analysis

We performed separate but similar analyses for larvae and adults and used either % black or black density as our dependent variables. We used analysis of variance (SPSS, 2004) to test the effects of monarch source

population, temperature treatment, sex (for adults only), and their interactions on either the percentage of black or the intensity of black of larvae and adults. In each case, full-sib family was treated as a random effect nested within the source population (full ANOVA model: melanism = population + sex + temperature + population*sex + population*temperature + sex*temperature + population*sex*temperature + family(population) + temperature*family(population)). Because measures of both the density and the percentage of black appeared to be normally distributed we did not transform them prior to analysis (also see Davis et al., 2004). We explored model fit and simplification (Crawley, 2002) by first removing each nonsignificant ($P > 0.05$) interaction term and comparing Akaike information criteria corrected for small sample sizes (AICc) between the full model and the model with $k - 1$ terms. Interaction terms were excluded from a simplified model if (a) their removal resulted in a lower value of AICc, or (b) their removal resulted in a small increase in AICc of less than 4.0, or (c) if their associated p-value was greater than 0.2. Next, non-significant main effect terms were removed from the reduced model if they were not present in any significant interaction term and if their removal satisfied criteria (a)–(c) above. We reported results from a final analysis that included main effects, covariates, and interaction terms for those parameters that explained substantial variation in each response variable as determined by the model fitting exercise. Comparison of means was performed where appropriate using Tukey's post hoc tests at the 0.05 level. Finally, we examined the correlation between measures of adult and larval melanism within each population and temperature treatment using data averaged across each family.

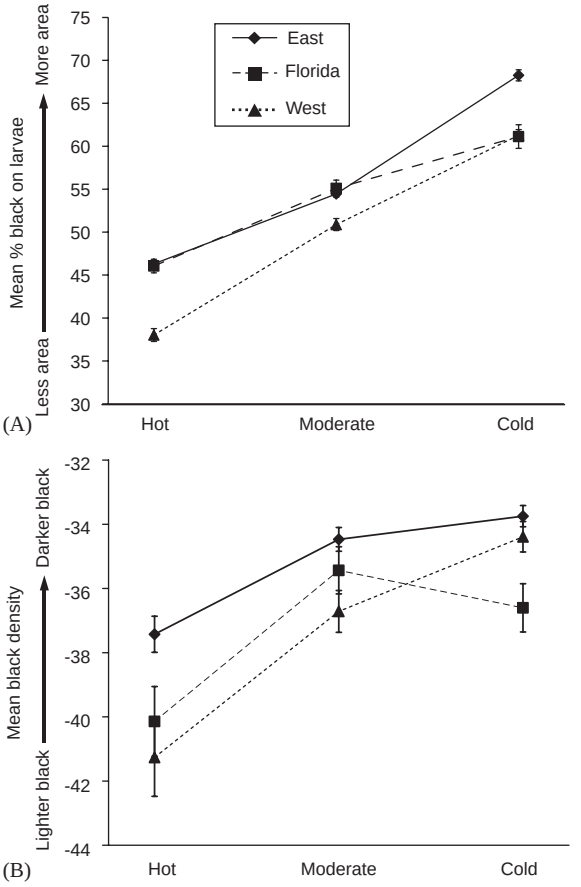

Fig. 2. Average percent black (A) and density scores (B) of larvae across all temperature treatments and populations. Error bars represent standard errors. Density scores shown in reverse order so that higher points on the Y-axis correspond to darker black coloration. Temperature treatments were 32, 26 and 19 °C.

3. Results

Although we initially started with 1065 first-instar larvae from a total of 29 females (six full-sib families from Florida, 10 families from the western population, and 15 families from the eastern population), only 688 larvae survived to fifth instar (and hence were able to be photographed). Similarly, due to mortality during the pupal stage and adult wing deformities, wing scans were obtained from a total of 552 adults (298 for the moderate treatment, 222 for the hot treatment, and 166 for the cold treatment).

3.1. Larval melanism

3.1.1. Temperature effects

Larvae from all populations were darker when reared in the cold temperature treatment and were lightest when reared under hot temperatures (Fig. 2). This main effect of temperature was highly significant for analyses of both the percentage of black on larval cuticles and the density of the black pigmentation (Table 1). Tukey's post hoc tests confirmed that the mean percentage of black for each of the three temperature treatments (hot = 44.2%, moderate = 53.9%, and cold = 64.9%) differed significantly from each other at the 0.05 level, and the direction and significance of comparison of means was similar for measures of black density (Fig. 2).

3.1.2. Population differences

Larval melanism levels differed among the three source populations as demonstrated by the highly significant main effect of population on the percentage of black coloration and on the density of black pigmentation (Table 1). In general, larvae from the eastern migratory population were the darkest and those from the western population were the lightest for both measures of larval melanism (Fig. 2). Tukey comparison of means based on the percentage of black showed that

Table 1
Results of ANOVA tests examining effects of temperature, population and full-sib family on larval melanism, measured as percentage of black or as black density

Dependent	Independent	df	F	p
% Black	Population	2	85.14	<0.001
	Temperature	2	449.54	<0.001
	Temperature*population	4	5.56	<0.001
	Mother (population)	26	4.96	<0.001
	Temperature*mother (population)	47	1.80	0.001
	Error	606		
Black density	Population	2	18.77	<0.001
	Temperature	2	62.01	<0.001
	Temperature*population	4	3.09	0.015
	Mother (population)	26	16.28	<0.001
	Temperature*mother (population)	47	13.50	<0.001
	Error	606		

Final models were obtained via comparison of model fit parameters (using minimum adequate model procedures based on AICc, and beginning with a full model with three factors and two two-way interaction terms). In these particular analyses, no terms were excluded based on criteria described in the Methods section in text.

eastern and S. Florida larvae had similar mean levels of dark pigmentation, but western larvae were significantly lighter (Fig. 2A). For black density, comparison of means showed that eastern larvae had significantly darker black pigmentation than western and S. Florida larvae, which were both similar in density (Fig. 2B).

Monarch larvae from each population responded differently to the temperature treatments, as demonstrated by the significant population by temperature interactions in analyses of both the percentage of black and the density of black pigmentation (Table 1). As indicated by Figs. 2A and B, eastern larvae became considerably darker in the cold temperature treatment relative to monarchs from the other populations. Meanwhile, western larvae were the lightest when reared under moderate and hot temperatures, but became as dark as or darker than S. Florida larvae when reared under cold temperatures. S. Florida larval melanism paralleled the eastern and western larvae in the hot and moderate temperatures, but Florida larvae had lower than expected responses to cold temperatures when measured using the black density score (Fig. 2B), as their levels of melanism were similar for both the moderate and cold temperature treatments.

3.2. Adult melanism

3.2.1. Temperature effects

Adult melanism was strongly influenced by rearing temperatures. We detected a highly significant main effect of rearing temperature on both the percentage of black and the density of black on adult forewings (Table 2). In terms of the density of black pigmentation, Tukey's post hoc tests demonstrated that adults from the cold treatment had the darkest or most opaque black (i.e. lowest density scores), and those from the hot treatment had the lightest black, and this effect was in the same direction for males and females (Figs. 3A and B). In terms of the percentage of black area on adult wings, males and females showed opposite responses to temperature treatments. Black area on female wings increased with increasing temperatures, whereas the area of black on male wings decreased with increasing temperatures (Figs. 3A and B). This interaction between sex and temperature on adult wing melanism was highly significant (Table 2).

3.2.2. Population differences

There was no main effect of monarch source population on the percentage or density of black coloration of adult forewings, nor were there population by sex interactions in either case. However, monarchs from different populations showed different responses to temperature in analyses of forewing density, as indicated by a significant population by temperature interaction effect (although observed differences were small; Figs. 3A and B), and a significant three-way interaction between sex, population and temperature (Table 2).

Finally, adult females responded to hot temperatures by increasing the relative area of black pigmentation on their wings, and we observed extreme cases of this effect in many western females reared in the hot temperature treatment (Fig. 3B). In several cases, the wings of these western females appeared "sooty," with dark-gray to black coloring extending into the orange areas of the wings (e.g., Figs. 4A vs. B).

Table 2
Results of ANOVA tests examining effects of temperature, population, sex, and full-sib family on adult wing melanism, measured as percentage of black or as black density

Dependent	Independent	df	F	p
% Black	Population	2	1.5	0.222
	Sex	1	2240.8	<0.001
	Temperature	2	6.08	0.002
	Sex*temperature	2	98.37	<0.001
	Mother (population)	35	5.37	<0.001
	Temperature*mother (population)	54	1.84	<0.001
	Error	455		
Black density	Population	2	1.23	0.294
	Sex	1	67.72	<0.001
	Temperature	2	449.15	<0.001
	Temperature*population	4	4.03	0.003
	Sex*temperature	2	9.92	<0.001
	Population*sex*temperature	6	2.77	0.012
	Mother (population)	35	1.88	0.002
	Temperature*mother (population)	50	1.99	<0.001
	Error	449		

Final models were obtained via comparison of model fit parameters (using minimum adequate model procedures based on AICc, and beginning with a full model with four factors, 4 two-way interaction terms and one three-way interaction term).

3.3. Family level effects and correlation between larval and adult traits

In all analyses we found highly significant effects of family origin (mother nested within population) on the percentage of black and the density of black on larvae and adults (Tables 1 and 2), indicating either strong maternal effects or a genetic basis for these traits. We also found highly significant interactions between temperature and mother (nested within population) in all tests, suggesting that families responded differently to each of the temperature treatments.

Finally, we found that measures of larval melanism were generally not correlated with melanism measures in adults. Although we could not pair individual larval measures with those from the same adults, we calculated family means within each temperature treatment for the following traits: larval percent area, larval black density, female percent area, female black density, male percent area, male black density. Bivariate correlations between these measures of adult and larval melanism were not significant when tested within each temperature treatment. It is important to note that when family means were examined across all treatments (Fig. 5A), there appeared to be a significant positive correlation between the percent black area in larvae and adult males ($n = 76$, $r = 0.56$, $p < 0.001$), and a significant negative relationship between larval and adult female percent area ($n = 74$, $r = -0.53$, $p < 0.001$; Fig. 5B), but within temperature treatments, there was no relationship between these traits. In comparisons of larval and adult black density, we found a significant positive relationship between family means for larval and adult females within the moderate temperature treatment ($n = 26$, $r = 0.48$, $p = 0.014$). Family means of adult female and adult male black density scores were also positively correlated within the moderate ($n = 27$, $r = 0.43$, $p = 0.027$) and cold temperature treatments ($n = 23$, $r = 0.43$, $p = 0.039$). Finally, when all family means were pooled across temperatures, there were positive relationships between larval and adult male density scores ($n = 76$, $r = 0.28$, $p = 0.014$), between larval and adult female density scores ($n = 74$, $r = 0.31$, $p = 0.008$), and between adult male and adult female black density scores ($n = 73$, $r = 0.918$, $p < 0.001$).

4. Discussion

4.1. Population differences

Results of this study demonstrated that the development of dark pigmentation in monarch butterflies responded strongly to rearing temperatures, with cooler temperatures generally inducing greater levels of melanism in both larvae and adults. We also showed that monarchs derived from three North American populations differed in their levels of thermally induced melanization. As expected, monarch larvae from the eastern migratory population were generally the darkest and became extremely melanic in the cold temperature treatment. Increased melanization allows insects to

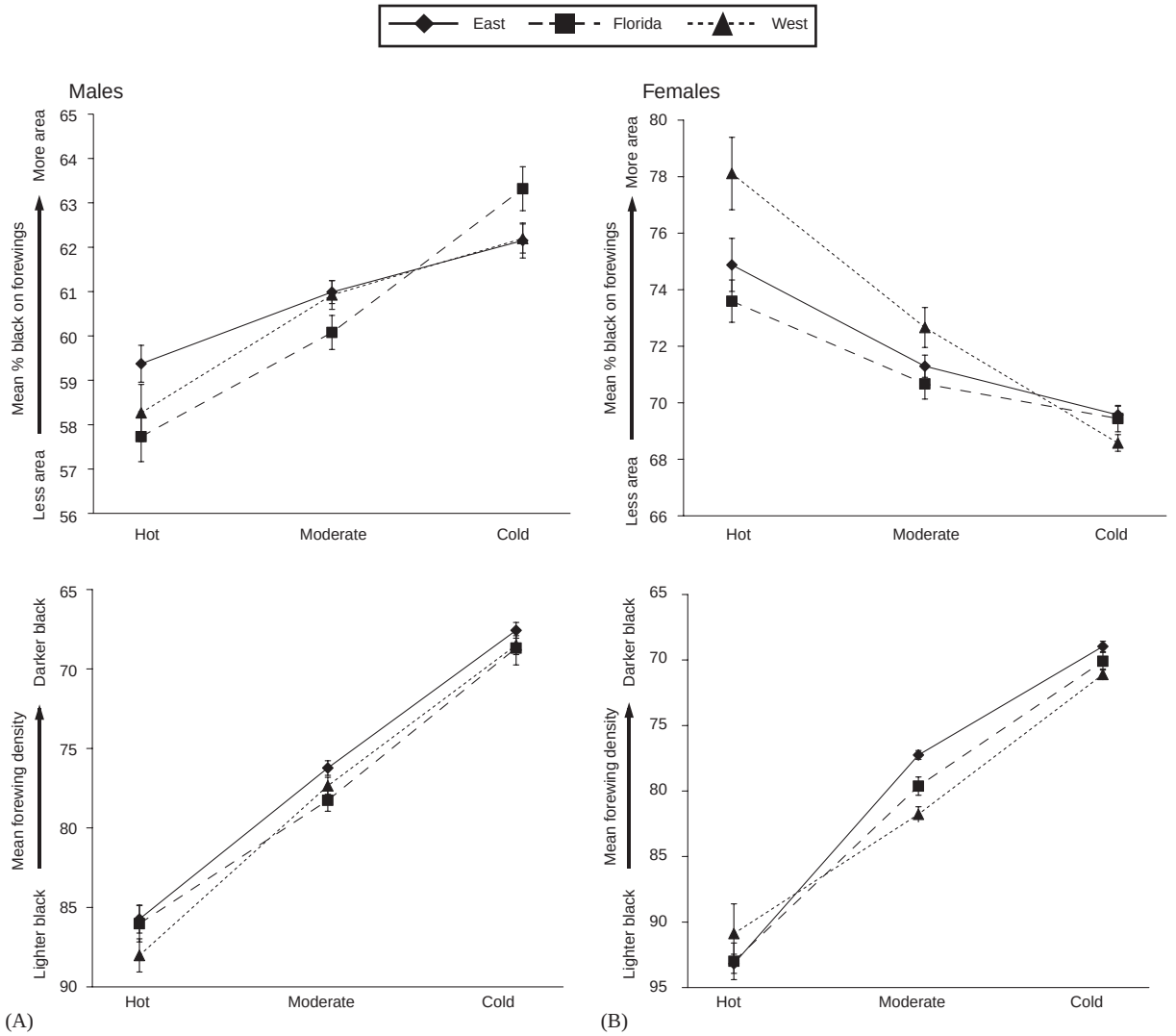

Fig. 3. Mean forewing % black and density scores of males (A) and females (B) from all temperature treatments. Error bars represent standard errors. Density scores shown in reverse order so that higher points on the *Y*-axis correspond to darker black coloration. Temperature treatments were 32, 26 and 19 °C.

develop more rapidly in cooler environments (Windig, 1999), and this trait would certainly be advantageous during the cool spring weather encountered in the early stage of this population's spring remigration (Howard and Davis, 2004). By comparison, western larvae were the lightest overall, particularly in the hot temperature treatment. Thus, western larvae would appear to be suited for tolerating hot climates such as those experienced in portions of the western United States during midsummer.

Unlike eastern and western caterpillars, monarch larvae derived from S. Florida showed no increase in black density in the cold temperature treatment. This is consistent with the observation that this population inhabits the mildest year-round climate and might not be exposed to extremely cool temperatures. However, our finding that larvae derived from S. Florida were not the lightest overall was unexpected in light of their warm native climate. One possible reason for this observation could be that high gene flow between the S. Florida and eastern migratory populations has limited the divergence of these two populations. Brower (1995) speculated that migrating eastern adults might travel south along the east coast in the fall and end up in Southern Florida, where they could overwinter in some years. Indeed, we now know that migrating eastern adults also intermix with resident adults in Cuba (Dockx et al., 2004), and it is not unlikely that this occurs in S. Florida as well.

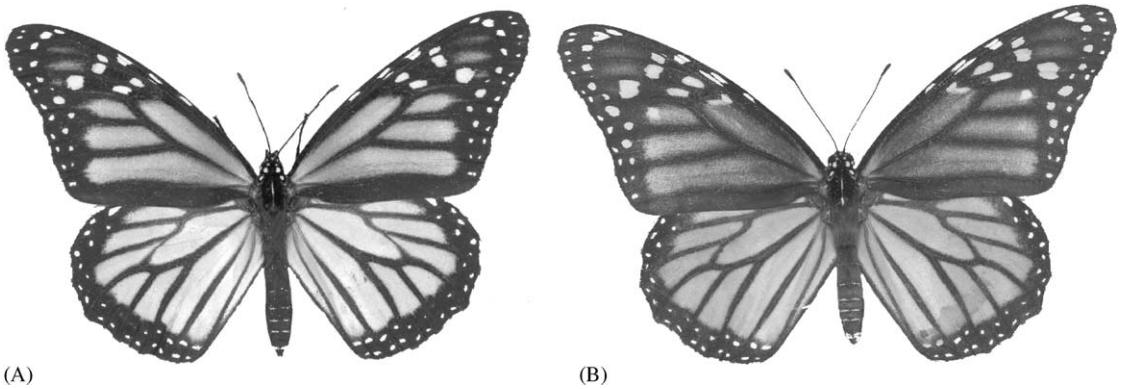

Fig. 4. (A) Typical western female monarch from the control temperature treatment, and (B) dark or "sooty" colored western female from the hot temperature treatment. Note the differences in the black density (i.e. the degree or shade of black color) in the wing margins and veins, as well as the greater area of black expressed as gray or black color in the orange cells of the sooty female.

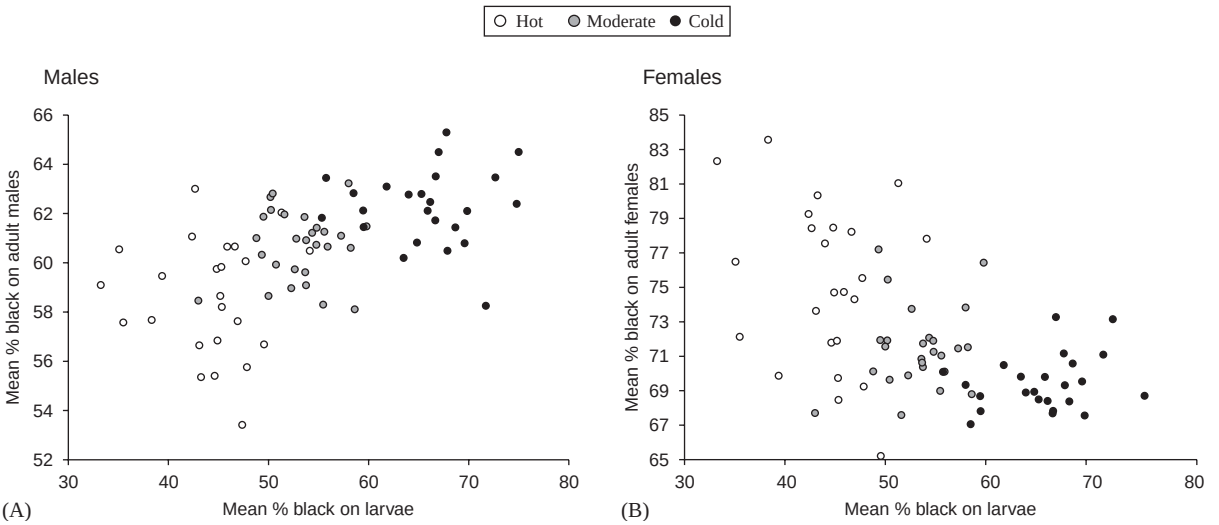

Fig. 5. Family means plotted for the mean percentage of black coloration on larvae (*X*-axis) and either adult males (A) or adult females (B) on the *Y*-axis. Each point is the mean percentage for a family based on progeny collected from eastern, western and S. Florida parents, calculate separately for each temperature treatment (as indicated by the colored points: open circles = 19 °C, gray circles = 26 °C and black circles = 32 °C).

Another possible reason why Florida monarchs were not as light as we expected, given the high temperatures they live in, is the fact that this population also is highly parasitized by the protozoan parasite, *Ophryocystis elektroscirrha* (Altizer et al., 2000). Although we took steps to ensure that all monarchs used in this experiment were parasite free, it may be that the naturally high exposure to this parasite has elevated the immune defenses of monarchs in the Florida population. Because immune function has been shown to correlate positively with increased melanization and body coloration in other insect species (Wilson et al., 2001), future experiments should examine the relationships between melanism, temperature, and parasite infection in this system.

Among adult butterflies, males from S. Florida were generally the lightest and males from the eastern migratory population were generally the darkest, consistent with expectations based on temperatures experienced in their native habitats. However, counter to our predictions, S. Florida females showed similar measures of melanization to those of eastern females across all temperature treatments. Perhaps the most surprising result of this study was our observation that adult females, especially western females, responded to hotter temperatures during development by also increasing the

overall area of dark coloration on their wings. As shown in Fig. 4, this resulted in a smudging effect of the color patterns on the wings, making the overall appearance, especially of western females, look "sooty." This result might represent a non-adaptive response to thermal stress, and in fact many individuals did not survive to adulthood in this hot treatment, although we did not observe a similar effect in males. On a related note, although our two methods of measuring melanism were generally in agreement, this was a situation where each measure was needed to describe the two different aspects of wing melanization among adult females. On the one hand, our percent black measure appeared to indicate that females were darker in hot temperatures, but measures of black density showed that the black coloration was actually less dense and appeared to be almost gray in appearance.

4.2. Heritability of melanism

In general, we found significant family-level effects on measures of larval and adult melanism, indicating a genetic basis for these traits or potentially strong maternal effects. This is consistent with previous work that showed joint contributions of underlying genetic effects and phenotypic plasticity to variation in melanism and other thermally relevant traits (Kingsolver and Huey, 1998). However, with a few exceptions, we found no strong evidence that measures of melanism in larvae were correlated with adult wing color, suggesting that underlying processes affecting these two traits might operate independently.

4.3. Image analysis in monarch research

Throughout this study, we noticed that much of the variation we observed between populations or temperature treatments in melanism measures was subtle. In fact in most cases, the differences we found were nearly indistinguishable to the unaided eye. For example, western larvae had approximately 50% of their bodies covered with black pigmentation (overall average) whereas eastern larvae had approximately 55% black coloring overall. Similarly, S. Florida males had about 2% less black coloration on their wings than eastern and western males. Although seemingly small, the differences were highly significant in statistical analyses. Thus, results reported here emphasize that this study could not have been completed without the use of our computer-assisted image analysis method, which can detect these fine-scale differences between colors (Davis et al., 2004). In fact, there are many questions that these techniques allow us to investigate, such as how measures of melanism and wing coloration influence male mating success (Solensky and Davis, in review), in addition to links between immune defenses, parasite resistance and body coloration in monarch butterflies.

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